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Studies on Hepatic Uptake of Antigen

I. COMPARISON OF INFERIOR VENA CAVA AND PORTAL VEIN ROUTES OF IMMUNIZATION

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Summary. An experimental animal model is described in which small injections of antigen (SRBC) into the portal venous system of the rat produces a reduced immune response in comparison with that obtained following similar injection into the inferior vena cava. The difference in humoral response, measured by circulating haemagglutinins and haemolysins was only detectable after repeated doses of antigen. The dose of SRBC necessary to demonstrate this difference is shown to be critical. Cell-mediated immunity—determined by measuring ear swelling 24 hours after intra-auricular injection of antigen—could be demonstrated in animals immunized via the inferior vena cava but not in those immunized via the portal vein.

Labelling of the antigen with radioactive chromium (^{51}Cr) showed that the increased immune response to inferior vena cava injected antigen was accompanied by a reduced hepatic uptake of antigen, but no significant alteration in splenic uptake.

INTRODUCTION

Although the liver is the largest organ in the reticuloendothelial system and is capable of sequestering large amounts of antigen, relatively little is known about the *in vivo* functional role of this organ in the immune response. The anatomical position of the liver is such that all antigens absorbed into the portal venous system from the gastro-intestinal tract pass through it. Previous studies have suggested that the immune response to orally administered antigens may differ from that obtained following antigen administered by other routes, and that this may be due to the effect of the liver.

Specific immunological unresponsiveness to dinitrochlorbenzene (DNCB) and to picryl chloride has been induced in adult guinea-pigs by prior feeding of these haptens (Chase, 1946, 1959). Other workers (Chase and Battisto, 1959; Battisto and Miller, 1962a,b; Coe and Salvin, 1963; Pomeranz, 1970) have confirmed these observations. The role of the liver in this phenomenon—known as the Chase-Sulzberger effect—was clearly demonstrated by Cantor and Dumont (1967), who showed that oral feeding of

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DNCB to adult dogs before subcutaneous injection of the hapten suppressed the formation of specific circulating antibodies, and that this effect could be abolished by prior diversion of portal blood from the liver. They suggested that the Kupffer cells in the liver might sequester antigen rather than process it for antibody formation with resultant immunological unresponsiveness to the antigen. Indirect evidence in support of this hypothesis is provided by the finding of raised antibody titres to gut-associated bacteria in patients with liver disease (Triger, Alp and Wright, 1972) and following porta-caval anastomosis (Bjørneboe, Prytz and Ørskov, 1972).

We have attempted to examine the role of the liver more directly by comparing the immune response to a particulate antigen, sheep red blood cells (SRBC) administered by the portal vein with that administered by the inferior vena cava—both injections taking place at laparotomy. The antigen uptake by various organs following immunization by these routes has been studied using ^{51}Cr-labelled SRBC.

MATERIALS AND METHODS

Animals

Random bred male Wistar rats (Scientific Farms Ltd, Ash, Kent) weighing 150–200 g were used except in one experiment when an inbred strain (Agus rats bred in the Nuffield Department of Medicine, Radcliffe Infirmary, Oxford) was used. All animals were fed on Oxoid 41 B Laboratory Animal Foodstuff and water *ad libitum*.

Antigen

Fresh, sterile, whole, defibrinated SRBC (Oxoid Limited (SR 51)) were stored at 4° and used a standard 2–3 days after preparation. The sheep erythrocytes were suspended in isotonic saline after washing three times in the same solution. The concentration was adjusted to a standard volume (0·5 ml), and the number of sheep red cells injected was measured by counting an aliquot of the suspension in a Coulter counter.

Injection

All animals were anaesthetized with ether alone. A midline abdominal incision was made and the viscera were exposed. The portal venous system was injected through a constant mesenteric vein running parallel to the small intestine in the region of the ileocolic junction and the inferior vena cava, directly at or above the level of the renal vein. The SRBC were drawn up in a 1·0 ml tuberculin syringe and delivered through a 25-gauge needle. This was given slowly over 10–15 seconds, with frequent admixture to minimize the possibility of 'streaming' in the portal venous system. At the end of injection the needle was rapidly withdrawn and haemostasis usually secured without haematoma formation by gentle pressure with a cotton wool swab. The viscera were replaced in the abdominal cavity and the wound was sutured in layers using 3/0 mersilk.

Repeated operations were carried out in the same manner, usually through the original incision. The second dose of antigen was administered by the same route as the first dose 14 days after the first immunization. In experiments in which a third dose was given, this was injected 14 days after the second. Complications were few and minor, but occasional animals were excluded because of the development of dense adhesions or because bleeding or haematomata developed as a result of the injections.

Bleeding

All animals were bled under ether anaesthesia by simple incision of the tail vein with a No. 11 surgical blade (Swann-Morton). In some animals up to fifteen samples over a period of 2 months were obtained. The blood was allowed to clot at room temperature and the clot was ringed before being stored overnight at 4° and centrifuged to separate the serum. Sera were stored at −20° and complement was inactivated by heating to 56° for 30 minutes before being tested for antibodies.

Antibody determination

Haemolysins. Haemolysin titres were determined by a microtechnique, in which 0·025 ml of serial dilutions of serum with Barbitone diluent (Oxoid Limited, BR 16) was incubated at 37° for 2 hours with equal volumes of freshly prepared 1 per cent solution of sheep red cells in Barbitone diluent and complement (Wellcome preserved guinea-pig serum VD 11 diluted 1 : 8 with isotonic saline) in microtitre plates (V-plates, Cooke Engineering Co., Alexandria, Virginia). The end-point was taken as that well which showed <50 per cent lysis. Titres were expressed as $\log_2$ of the reciprocal of the end-point dilution.

Haemagglutinins

These were determined using a similar microtechnique. Antigen (1 per cent SRBC in Barbitone diluent) was mixed with serial dilutions of serum. The plates were incubated at 4° overnight before reading, the end-point being taken as that well which showed less than 50 per cent agglutination. Titres were expressed in the same way as described for haemolysins.

2-*Mercaptoethanol extraction*

IgM haemagglutinating antibodies were extracted from the serum using a modification of the method described by Mollison (1967). Serum was mixed with equal volumes of 0·1 M 2-mercaptoethanol in phosphate-buffered saline, pH 7·2 and incubated at 37° for 10 minutes. One per cent sheep red cells in barbiturate buffer was then added and the mixture left overnight at 4°. Equal volumes of serum, phosphate-buffered saline and sheep red cells were incubated together as a control.

Delayed hypersensitivity

This was measured using a modification of the method described by Axelrad (1968), the antigen being injected into the ear of the rat rather than into the hind paw. In the mouse the measurement of ear swelling following local application of a sensitizing agent has been used extensively as an index of delayed hypersensitivity (Asherson and Ptak, 1968). A sensitizing dose of 0·5 ml of 0·2 per cent solution of sheep red cells in normal saline was injected into the tail vein under ether anaesthesia. Ten days later 0·05 ml of a 7·5 per cent solution of sheep red cells in isotonic saline was injected into the left ear of the non-anaesthetized animal—using a 30-gauge needle in such a way as to produce the maximum area of swelling at a point near the edge of the ear just posterior to its tip. The right ear was left untouched and was used as a control. The thickness of both ears was measured before injection, 4 hours after injection and 24 hours after injection using an engineer's micrometer which read accurately to 0·05 mm (Dial Gauge Model Quick test A 02 T, Carobronze Limited, London, W.4). Preliminary experiments using a urea extract of the

antigen (Boyden, 1964) showed that this produced identical ear swelling at 24 hours compared with the crude antigen, and so the simple SRBC preparation was used instead.

Immunization with labelled antigen

SRBC were washed three times with isotonic saline, and diluted with 2–3 ml of saline before the addition of radioactive chromium in order to minimize possible damage on exposure to the radioactivity. Two hundred microcuries of [^{51}Cr]sodium chromate (The Radiochemical Centre, Amersham) was added and the mixture circulated for half an hour at room temperature. The [^{51}Cr]SRBC were then washed three times with isotonic saline before dilution with saline, kept at 4° until use in order to minimize haemolysis, and injected within 4 hours of preparation.

Doses varying from 10^5 to 3×10^9-labelled SRBC were injected via the inferior vena cava in order to demonstrate the effect of antigen dose on the tissue uptake and distribution. For the experiments comparing the distribution of antigen following the two routes of immunization 10^6 [^{51}Cr]SRBC were injected, since this dose of unlabelled antigen was shown to produce differences in immune response after repeated injections by the different routes.

The immunization experiments described here concern two groups of animals—termed 'non-immune' and 'immune'. Non-immune animals are defined as those animals which had not previously been exposed to SRBC, while immune animals had received two doses of non-radioactive-labelled SRBC by either inferior vena cava or portal vein route before the third injection of labelled antigen, which was administered by the same route as the first two injections.

Radioactive counting

All animals were killed by ether anaesthesia and organs removed by simple dissection. Liver, spleen and both kidneys were routinely counted. The tissues were counted by placing in small plastic disposable tubes suitable for insertion into a well counter. The spleen and both kidneys (separately) were small enough to be placed *in toto* in a single tube, while the liver was cut up into small pieces such that the volume of each was comparable to that of the other tissues and each aliquot placed in a separate tube for counting (usually eight or nine tubes were necessary for one whole liver). The distribution of radio-isotope in the liver was so uneven that counting the whole liver was the most accurate way to determine the uptake.

Counting was performed in a Wallac gamma sample counter. Each tube was counted for a sufficient length of time to ensure a maximum of 3 per cent counting error.

RESULTS

CIRCULATING ANTIBODY RESPONSE

Fig. 1 shows the primary and secondary immune response to 10^7 and 10^8 SRBC injected via the inferior vena cava compared with the portal vein. No significant difference between the two routes of immunization is detectable. The results obtained for the haemolysin antibody response were similar to the haemagglutinins throughout this study, and the latter have been omitted for the sake of brevity.

The antibody response to 10^6 SRBC injection is shown in Fig. 2. No difference in primary immune response between the two routes of immunization is detectable, but the

antibody response to a second inferior vena cava injection is higher than that produced by the portal vein injection. The difference is enhanced by a third injection and is statistically significant.

10^5 SRBC produces no significant differences in primary immune response between the

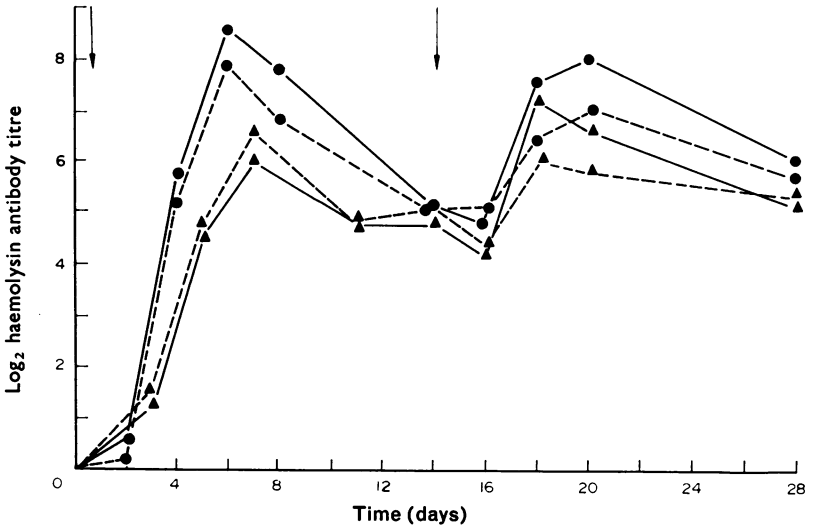

FIG. 1. Primary and secondary immune response to inferior vena cava and portal vein injection of 10^8 and 10^7 sheep red cells (haemolysin antibody). ●——●, 10^8 SRBC inferior vena cava injection. ●- - -●, 10^8 SRBC portal vein injection. ▲——▲, 10^7 SRBC inferior vena cava injection. ▲- - -▲, 10^7 SRBC portal vein injection. Five animals in each group.

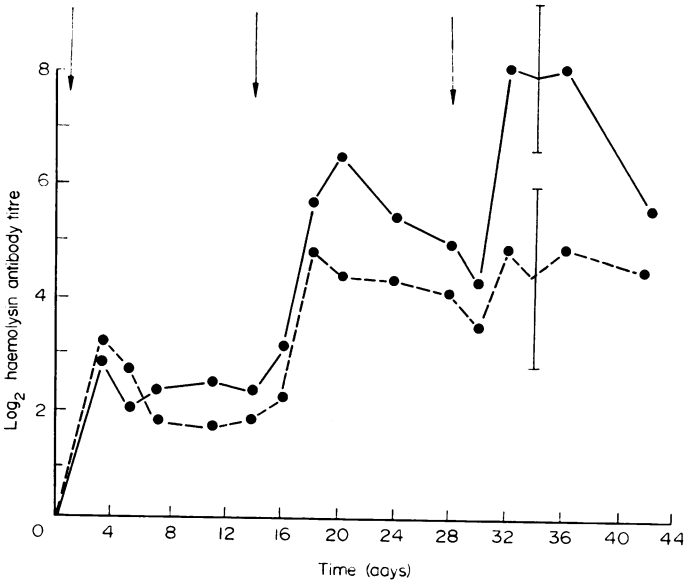

FIG. 2. Primary, secondary and tertiary immune response to inferior vena cava and portal vein injection of 10^6 sheep red cells (haemolysin antibody). ●——●, 10^6 SRBC inferior vena cava. ●- - -●, 10^6 SRBC portal vein. Fourteen animals in each group.

two routes of immunization, and although the antibody titres following inferior vena cava immunization are slightly higher than those following portal vein immunization, the results are not statistically significant.

2-MERCAPTOETHANOL EXTRACTION

Extraction of IgM antibody with 2-mercaptoethanol (2-ME) shows that very little 2-ME-resistant antibody contributes to the maximum titres found after the second or third injection—a finding previously noted by Cooper and Turner (1968). Similar amounts of 2-ME resistant antibody were found in the serum of animals immunized by the portal vein route compared with those immunized by the inferior vena cava.

TABLE 1

24-HOUR RAT EAR SWELLING FOLLOWING INTRA-AURICULAR INJECTION OF $10^5/10^6$ SHEEP RED CELLS

Route of antigen administration	Number of sheep red cells ($\times 3$)	Number of animals	Per cent increase in ear thickness	(cf. P controls)
Inferior vena cava	10^6	14	28·4	$0{\cdot}01 < P < 0{\cdot}05$
Inferior vena cava	10^5	4	28·4	$0{\cdot}01 < P < 0{\cdot}05$
Portal vein	10^6	12	8·4	N.S.
Portal vein	10^5	3	12·3	N.S.
Controls (i.e. no antigen)	0	5	6·9	—

DELAYED HYPERSENSITIVITY

The mean increase in ear thickness at 24 hours following intra-auricular injection of sheep red cells is shown in Table 1. The increase in ear thickness in rats immunized by three inferior vena cava injections of antigen is compared with those given three portal vein injections of antigen and controls (rats given three inferior vena cava injections of normal saline under comparable conditions). No difference was demonstrable between the controls and the animals immunized with SRBC via the portal vein, while rats immunized with SRBC via the inferior vena cava showed a significant increase in ear thickness.

ORGAN UPTAKE OF [^{51}Cr]SRBC

The percentage uptake of labelled SRBC by liver, spleen and kidney following a range of doses of SRBC injected i.v. into non-immune animals (via the inferior vena cava) is shown in Fig. 3. All animals were killed 2–4 hours after the i.v. injection. Doses lower than 5×10^5 [^{51}Cr]SRBC produced such low tissue counts that accurate estimation of radioactivity was impossible, while doses above 3×10^9 SRBC could not be injected in a volume of 0·5 ml.

The percentage uptake by the liver is constant at about 60 per cent over the range 5×10^5–10^7 SRBC, but this falls off with increasing doses of SRBC. The percentage uptake by spleen and kidneys does not vary significantly over the range of doses studied.

ORGAN UPTAKE FOLLOWING INFERIOR VENA CAVA AND PORTAL VEIN ADMINISTRATION OF [^{51}Cr]SRBC (Table 2)

The uptake by liver, spleen and kidneys 2 hours after injection of labelled SRBC shows no significant difference between the two routes of immunization.

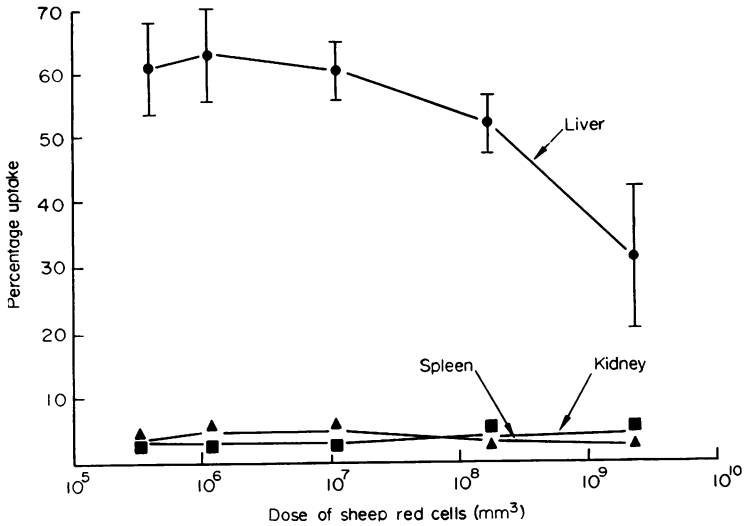

FIG. 3. Percentage uptake of ^{51}Cr-labelled sheep red cells by liver with increasing doses of sheep cells (inferior vena cava route).

TABLE 2

PERCENTAGE ORGAN UPTAKE OF 10^6 [^{51}Cr]SRBC FOLLOWING INFERIOR VENA CAVA AND PORTAL VEIN INJECTION

	Liver	Spleen	Kidney
Non-immune rats			
Portal vein injection	62·0±8·5	2·9±0·6	3·1±0·3
Inferior vena cava injection	66·1±8·1	3·8±1·4	2·2±0·9
Immune rats			
Portal vein injection	55·9±9·0	2·6±1·2	2·4±1·1
Inferior vena cava injection	39·4±5·6	4·5±1·5	3·4±0·7

Eight rats in each group

Immune rats.

A marked difference between the two routes of immunization is apparent when labelled SRBC are injected into rats previously immunized via the inferior vena cava or portal vein. While the hepatic uptake following portal venous immunization falls only slightly ($P>0·05$) the uptake of the liver following inferior vena cava immunization is considerably reduced. This is accompanied by a small increase in the uptake by the spleen, which was not statistically significant.

DISCUSSION

The present study shows that repeated injection of small amounts of SRBC into the portal vein resulted in a lower circulating antibody titre than did the injection of equivalent amounts of antigen into the inferior vena cava. The primary immune response

showed no difference between the two routes. In addition a significant difference in ear thickness 24 hours after intra-auricular injection of SRBC was noted in animals immunized with SRBC via the inferior vena cava compared with those given portal vein antigen. It is unlikely that these differences can be explained on technical grounds. Leakage of antigen might be more likely to occur following injection into the smaller mesenteric vessels, but in one control experiment (data not shown) the antibody response to repeated doses of SRBC injected extravascularly into the mesentery is higher than that following mesenteric venous injection. Other factors such as retrograde hepatic venous flow, intrahepatic shunting and individual variability in animals would tend to minimize the differences between the two routes of immunization. With regard to the last variant, a single experiment comparing the immune response to SRBC in rats from a pathogen-free syngeneic inbred strain (Agus) showed the same difference between the two routes of injection but the individual variation within each group was as great as in the non-inbred strain.

The possibility that the reduced immune response following repeated portal vein immunization might be due to sequestration of antigen by the liver is supported by the results of the organ distribution of radio-labelled antigen. In the non-immunized animal the percentage uptake of labelled SRBC by the liver, spleen and kidneys is the same regardless of route of administration. Animals repeatedly immunized via the inferior vena cava show a significantly reduced hepatic uptake compared with the portal vein group. This reduction accompanies an increased antibody response, but an increased splenic uptake is not found. The percentage antigen recovery from the repeated inferior vena cava immunized rats is much lower than the portal vein immunized animals. Some of this may be due to increased renal clearance, possibly occurring secondary to immune lysis of antigen within the blood stream before the antigen can become lodged within organs such as the liver, although no significant increase in renal count has been detected. Antigen may also be lodged in other organs but in quantities too small to detect by the technique used, since attempts to count lung and marrow from these animals were unsuccessful.

Antigen dose appears to be critical in demonstrating a difference between the immunizing routes. The liver removes a fixed proportion of labelled SRBC over a dose range 10^5–10^7 cells, this percentage falling with larger doses of antigen. In contrast to the studies of Souhami (1972) this is not accompanied by an increase in splenic uptake, although this might be due to a species difference. Following immunization with the lower doses, the liver may act by removing a critical amount of portal vein injected antigen before it can reach the rest of the reticuloendothelial system. Using larger doses of sheep cells the 'spill-over' increases to such an extent that sufficient antigen becomes available to the antibody-producing cells for an adequate response irrespective of the sequestering properties of the liver.

A significant difference in ear thickness 24 hours after intra-auricular injection of SRBC has been noted in animals immunized with 10^6 SRBC via the inferior vena cava compared with those given portal vein antigen. Similar differences have also been demonstrated following immunization with 10^5 SRBC, possibly suggesting that the liver may play a role in the mechanism of cell-mediated immunity beyond merely regulating the amount of antigen made available to the extra-hepatic tissues. There was no correlation between the extent of ear swelling and the level of circulating antibody response, a disparity previously noted by Axelrad (1968) using foot swelling as an index of delayed hypersensitivity.

If the reduced immune response in portal vein immunized animals is due to hepatic sequestration, the liver macrophages—in contrast to other macrophages—must be capable of taking up and processing antigens without initiating the process of antibody formation. Miller and Bale (1954) using ^{14}C-labelled plasma proteins, have been unable to show that γ-globulin is formed in the liver in contrast to the spleen and lymph nodes, implying that even if antigen processed by the Kupffer cells leads to the production of antibody, the latter is not produced locally. This is supported by the studies of Franzl (1962), who has shown that mouse liver homogenates containing antigen (SRBC) on injection into sensitized mice failed to elicit an immune response, whereas equivalent amounts of splenic-sequestered antigen result in significant antibody production. Similar findings have been reported by Inchley using T^4 bacteriophage.

The differences between portal vein and inferior vena cava administration of sheep erythrocytes could be explained in immunological terms by postulating that the sequestration of antigen by the liver affects the antigen delivered to the reticuloendothelial system in such a way as to produce a system of low-dose tolerance (Dresser and Mitchison, 1968). The experiments using the sheep erythrocyte system have stressed the importance of antigen dosage, a fact which is not apparent in the Chase-Sulzberger phenomenon because of the variability of antigen uptake from the intestine. Mitchison and Taylor (1971) have shown that low-dose tolerance to soluble antigens selectively affects thymus derived (T) lymphocytes so that T and B cell co-operation in the production of antibodies is depressed.

It is of interest to note that Playfair & Purves (1971), in a study of T and B cell co-operation in irradiated mice, found that maximum cell co-operation in the formation of antibody to sheep erythrocytes occurred with a dose of 10^6 sheep red cells. This corresponds to the dose of antigen used in the experimental animal model described here to demonstrate a difference between portal vein and inferior vena cava administered antigen, and it is conceivable that in this situation the intact liver may be able to sequester sufficient antigen in order to disturb this process of cell co-operation.

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